Regulation of Diacylglycerol Acyltransferase in Developing Seeds of Castor

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ABSTRACT: We have previously reported the cloning of castor diacylglycerol acyltransferase (RcDGAT) based on its homology to other plant type 1 diacylglycerol acyltransferases (DGATs). To elucidate the physiological role of the RcDGAT, we have investigated the regulation of RcDGAT expression in developing seeds of castor. The RcDGAT transcript appeared at 12 d after pollination (DAP), reached the highest level at 26 DAP, and declined rapidly after that. However, the RcDGAT protein started to accumulate at 26 DAP, reached its peak at 47 DAP, then remained at this high level until 54 DAP. The significant difference between the expression of mRNA and protein indicates that gene expression of RcDGAT in maturing castor seeds is controlled at the posttranscriptional level. We found that DGAT activity measured in microsomal membranes isolated from seed at different stages of development was parallel to RcDGAT protein level, suggesting DGAT activity is mainly a function of the level of RcDGAT protein. We monitored the triacylglycerol (TG) composition and content during seed development. Compared with the overall rate of TG accumulation, DGAT activity appeared coincidently with the onset of lipid accumulation at 26 DAP; the highest DGAT activity occurred during the rapid phase of lipid accumulation at 40 DAP; and a decline in DGAT activity coincided with a decline in the accumulation rate of TG after 40 DAP. The ricinoleate-containing TG content was very low (only about 7%) in oil extracted from seeds before 19 DAP; however, it increased up to about 77% of the oil at 26 DAP. The relative amount of triricinolein in oil at 26 DAP was 53 times higher than that at 19 DAP, and it was about 76% of the amount present in oil from mature castor seeds. The close correlation between profiles of RcDGAT activity and oil accumulation confirms the role of RcDGAT in castor oil biosynthesis.

Paper no. L9612 in *Lipids 39,* 865–871 (September 2004).

The oil from castor seed (*Ricinus communis*) contains 90% ricinoleate, a hydroxy FA that is particularly useful because it is extremely viscous and is therefore an excellent source for biodegradable lubricants and greases (1). Worldwide, the annual production of castor oil is about 460,000 tons (1.1 million tons of seeds), produced mainly in India, Brazil, and China (http://www.hort.purdue.edu/newcrop/). The United States spends over $50 million annually to import castor for industrial applications including such diverse products as lubricants, greases, plasticizers, cosmetics, pharmaceuticals, paints, plastics, coatings, antifungal compounds, shampoo, and thermopolymers.

Conventional domestic production of castor oil, however, is seriously hindered owing to the presence of a toxic protein, ricin, as well as various allergenic albumins. Efforts to engineer other plants to produce oil with high levels of ricinoleate by expression of the oleoyl-hydroxylase gene have been unsuccessful as a result of the plants' inability to "move" ricinoleate through the lipid biosynthetic pathway into triacylglycerols (TG) (2). An understanding of how plants assemble TG, and specifically how a plant like castor can accumulate seed oil in which the FA composition exceeds 90% in a single FA, ricinoleate, is a prerequisite before such an effort can be undertaken successfully. Moreover, such basic biosynthetic information will be useful in engineering microbes that can convert surplus oils to higher-value industrial oils. It will also prove useful in reprogramming castor to produce other useful FA.

The regulatory factors that influence oil accumulation in castor seeds are largely unknown (3). Acyl-CoA-dependent diacylglycerol acyltransferase (DGAT) is thought to be a key enzyme in controlling the biosynthetic rate of TG in most oilseeds (4,5). It is the only enzyme in the Kennedy glycerol-3-P pathway that is exclusively committed to TG synthesis (6) although there are two types, DGAT1 and DGAT2. The DGAT is responsible for the acylation of 1,2-diacylglycerol (1,2-DG) at the *sn*-3 position using an acyl-CoA substrate. Genes encoding DGAT have been cloned and characterized from several plant species (7–9). Both DGAT1 and DGAT2 have been reported in plants. While the DGAT2 from fungi has been shown to have a stronger preference for acylating DG with medium-chain acyl groups, it does not apparently have a significant effect on the FA composition of the oil (10). We recently reported the identification of a cDNA encoding a castor DGAT (RcDGAT) and demonstrated that the RcDGAT preferentially incorporated diricinolein into TG (11). Determination of the content and composition of storage lipids in developing seeds has been reported in numerous oilseed crops (12–14); however, how the developmental profile of an enzyme participating in TG biosynthesis is regulated and correlated with oil accumulation remains poorly understood. In the present paper, we report our studies of the regulation of

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Abbreviations: AG, acylglycerols; DAP, days after pollination; DG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DIG, digoxigenin; PDAT, phospholipid:diacylglycerol transferase; RcDGAT, castor DGAT; TG, triacylglycerol.

RcDGAT protein expression and function during castor seed development. We also examine the correlation between RcDGAT activity and oil accumulation.

EXPERIMENTAL PROCEDURES

Northern blot analysis. RNA samples were extracted from castor seeds at different developmental stages using the method of Gu *et al.* (15). Five μg of total RNA was applied to each lane in a 1% agarose gel with 2% formaldehyde. The probe used for hybridization was generated by PCR labeling with digoxigenin (DIG-dUTP) using RcDGAT cDNA as template and the primers: 5′-AAGACCCCATGGCGATTCTC-GAAACGCCAGAA-3′ (HE-15F) and 5′-CTGAGAGCTT CAGAACCCTCTCAA-3′ (HE-6R). Northern analysis was performed based on the DIG Application Manual for Filter Hybridization (Roche Molecular Biochemicals, Mannhein, Germany).

Antibody production. Antibodies for RcDGAT were raised in two rabbits immunized with the peptide (CVLLYYHDLM-NRDGN), which corresponds to the C terminus of the native protein (Pacific Immunology Corp., Ramona, CA). The carrier protein, keyhole limpet hemocyanin, was conjugated to the N-terminal Cys of the peptide using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Prior to immunization, serum samples were obtained and the rabbits were injected four times at 3-wk intervals with the linked peptide. Serum was collected 1 wk after the final injection and column-purified by affinity chromatography. The titer was determined using the remaining peptide.

Total protein and microsomal preparations. Castor seeds from different developmental stages were harvested and homogenized in a buffer (16) containing 400 mM sucrose, 100 mM Hepes-NaOH pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, and a protease inhibitor cocktail tablet per 10 mL from Boehringer Mannheim (Indianapolis, IN). The homogenate was centrifuged at 10,000 × *g* for 10 min to remove cell debris, and the supernatant (total protein fraction) was spun again at 100,000 × *g* for 90 min. The pellet was resuspended in the homogenizing buffer (microsomal fraction), and the protein concentration was determined using Bradford reagent (BioRad, Hercules, CA). These microsomal preparations were stored at –80°C and used for western blot and DGAT activity assay.

Western blot analysis. Thirty micrograms of total or microsomal proteins from different developmental stages were separated by SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes. The blot was incubated with the polyclonal rabbit antibodies described above (at 1:5,000 dilution). Horseradish peroxidase conjugated goat anti-rabbit (Amersham Pharmacia, Piscataway, NJ) secondary antibodies were diluted 1:3,000. Horseradish peroxidase activity was visualized using chemiluminescence (ECL kit; Amersham Pharmacia).

In vitro *DGAT assay.* The DGAT assay was performed as in Cases *et al.* (17) with minor modifications. Assay mixtures (100 μL) contained 0.1 M Tris-HCl pH 7.0, 20% glycerol, 400 μM 1,2-diricinolein, and 20 μM [^{14}C]oleoyl-CoA (200,000 cpm), and reactions were started by the addition of 100 μg microsomal protein. The reactions were incubated for 15 min at 30°C with shaking and stopped by the addition of 1 μL 10% SDS. The 1,2-diricinolein was prepared as a 10-mM stock in 0.5% Tween-20. Lipids were extracted from assay mixtures using chloroform/methanol as previously described (18). The molecular species of TG products were separated using C18 HPLC (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman Instruments Inc., Fullerton, CA) (19). DGAT activity was determined based on the ^{14}C-label incorporated into the TG products from [^{14}C]oleoyl-CoA.

Quantification of RcDGAT protein and lipid contents. Total lipids were extracted from seeds by grinding in a 2:1 chloroform/methanol mixture (20). Following the addition of 0.9% NaCl, the chloroform phase was evaporated to dryness under a stream of N_2. Oil contents (%) were determined by dividing the extracted oil (g) by the sample's fresh weight. The abundance of RcDGAT proteins in seed samples was quantified by measuring the average intensity value of the protein bands on the western blot using Bio-Rad Quantity One Quantitation software. The relative amounts of oil content and RcDGAT protein were calculated by normalizing against the highest value as 100%.

HPLC. HPLC was carried out on a liquid chromatograph (Waters Associates, Milford, MA) using a flow scintillation analyzer (150TR; Packard Instrument Co., Downers Grove, IL) to detect [^{14}C]-labeled TG or an ELSD for mass quantification (MK III; Alltech Associates, Deerfield, IL) at a flow rate of 1 mL/min. The drift tube temperature of the ELSD was set at 80°C. The nitrogen gas flow of the nebulizer of the ELSD was set at 1.0 L/min. The nitrogen pressure on the regulator of the nitrogen tank was set at about 65 psi. Separation of lipid classes and molecular species of TG was performed as we reported previously (21,22). The quantification of different molecular species of TG in Table 1 was based on relative ELSD peak area (%) in the HPLC chromatogram. Our previous data indicated that ELSD responses of different amounts of the standards of molecular species of TG were nearly linear (22).

RESULTS AND DISCUSSION

Disparity of RcDGAT expression in mRNA and in protein. To investigate how the expression of RcDGAT is regulated in maturing castor seeds, Northern blot analysis was performed using RNA samples extracted from castor seeds at different developmental stages. Figure 1 shows that RcDGAT transcript was detectable at 12 d after pollination (DAP), reached a maximum at 26 DAP, and declined thereafter. Our previous results based on reverse transcription-polymerase chain reaction indicated that the highest transcript level appeared at 19 DAP; this discrepancy may be due to the variation in samples and detection methods used in the two experiments. To determine the expression pattern of RcDGAT protein, we raised

TABLE 1
Molecular Species of TG Identified and Their Contents (%) in Castor Oil at Different Stages of Seed Development[a]

TG species	19 DAP	26 DAP	61 DAP
RRR	1	53	70
RRLs	0	0.29	1.37
RRLn	0	2.92	1.14
RRL	5.71	9.22	9.26
RRO + RRP	0	10.2	6.51
RRS	0	0.99	0.69

[a]Abbreviations: TG, triacylglycerols; R, ricinoleic acid; Ls, lesquerolic acid; Ln, linolenic acid; L, linolenic acid; O, oleic acid; P, palmitic acid; S, stearic acid; DAP, days after pollination.

antibodies to a peptide of RcDGAT in rabbits. The synthetic peptide corresponded to a 15-amino acid peptide in the C-terminus of RcDGAT. Since the C-terminus of RcDGAT is not hydrophobic, this appears to be a suitable region from which to derive an epitope. Total proteins were extracted and analyzed with these antibodies by western blot. The protein started to appear at 26 DAP, kept increasing to 47 DAP, and remained at a high level to 54 DAP, then quickly decreased to a very low level at 61 DAP. The disparity of accumulation patterns between RcDGAT protein and mRNA was remarkable. Similar results were obtained from studies with cell cultures of oilseed rape (9). It could be that the mRNA for RcDGAT encodes a relatively stable protein because most of the protein is embedded in the membrane, and therefore resistant to endogenous proteases, or because some of the gene expression is regulated at the posttranscriptional level. The predicted size of the RcDGAT protein is 60 kDa; however, the polypeptide detected on the blot prepared from the total protein extracts is only about 50 kDa, which appears to be a product of posttranslational proteolytic processing of the native RcDGAT protein. The full-length RcDGAT protein was observed on the western blot prepared from microsomal samples isolated from 26 and 33 DAP (Fig. 2B), although it was

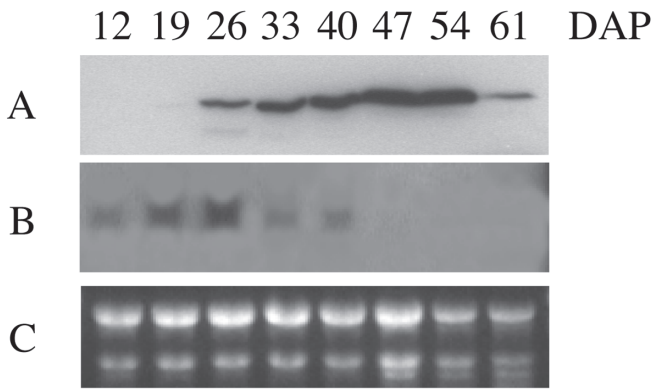

FIG. 1. Expression of castor diacylglycerol acyltransferase (RcDGAT) mRNA and protein. RNA samples were extracted from castor seeds at different stages of development from 12 to 61 d after pollination (DAP). Total RNA (5 μg) was used for northern analysis (B). Ethidium bromide staining is shown to demonstrate the equal loading of RNA in the blot (C). Another set of seeds was used to extract protein. Total protein (30 μg) was used for western blot analysis (A).

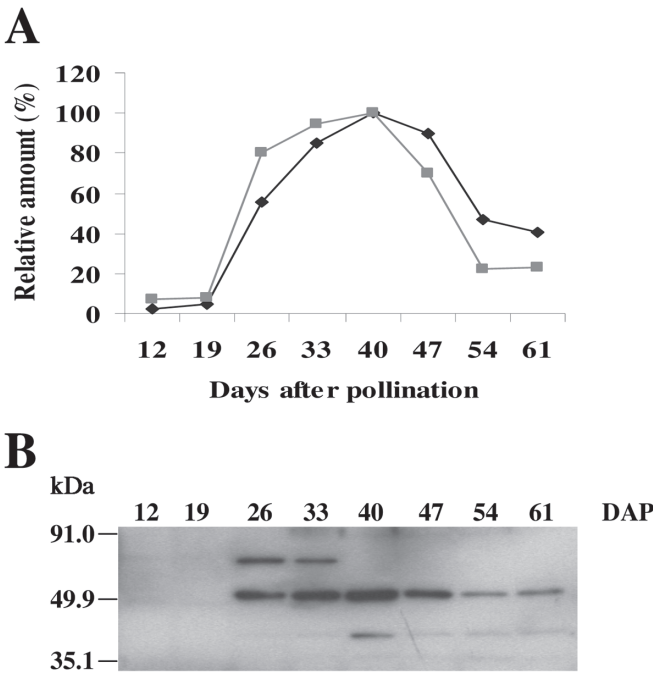

FIG. 2. Accumulation of RcDGAT proteins (■) and diacylglycerol acyltransferase (DGAT) activities (◆) during seed maturation (A). Microsomal fractions isolated from seeds at 12 to 61 DAP were used for DGAT assay and western blot analysis. The levels of RcDGAT protein and activity at 40 DAP were used for normalizing to 100% and calculating the relative amounts in other stages. The activity at 40 DAP was 412.72 pmol/min/ mg. The accumulation of RcDGAT protein in seed samples was quantified by measuring the average intensity value of the protein bands on the western blot (B) using Bio-Rad (Hercules, CA) Quantity One Quantitation software. Molecular masses of proteins are marked in the left margin, and 30 μg of microsomal protein was loaded in each lane on the blot. For other abbreviation see Figure 1.

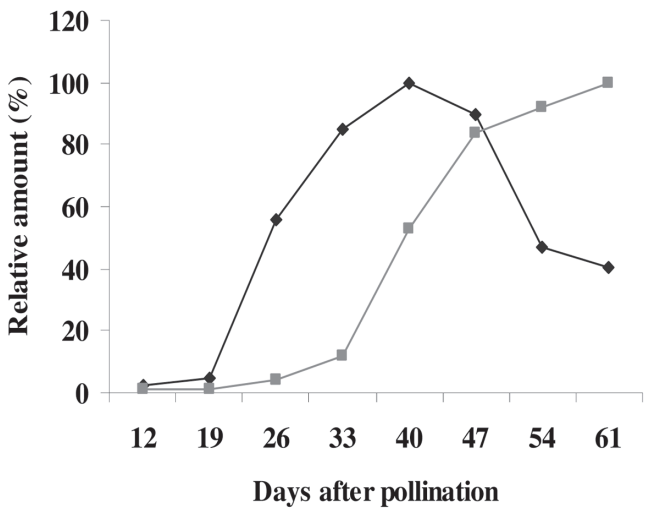

FIG. 3. Accumulation of oil (■) and DGAT activities (◆) during seed maturation. Extraction of lipids and determination of oil content in seed samples were performed as described in the Experimental Procedures section. The oil contents at 61 DAP (66% of the fresh weight) and the enzyme activity at 40 DAP (624 pmol/mg/min) were normalized to 100% to calculate the relative amounts in other stages. For abbreviations see Figures 1 and 2.

not the predominant form of the protein. Since RcDGAT is an integral membrane enzyme, most of the C-terminal region is embedded in the membrane (11), whereas the N-terminal region containing 119 amino acid residues is hydrophilic and thus accessible to proteases. Moreover, the antibody was raised against the C-terminal 15 amino acid residues of the RcDGAT protein, and it detects only protein containing the C-terminus of the protein. If the C-terminus is cleaved, there will be no signal on the western blot. The specificity of the antibody was confirmed by western blot of extracts from non-induced yeast and yeast induced to express RcDGAT protein (data not shown). Several groups have reported that DGAT activity was found in all particulate fractions prepared from developing seeds (23–25). We compared the RcDGAT levels in protein from total extracts and from microsomal fractions (Figs. 1A and 2B). The overall trends among multiple independent experiments showed that the accumulation patterns of RcDGAT protein from 12 to 40 DAP were identical in the two protein extracts but started to differentiate after 40 DAP. After 40 DAP, the protein level in the microsomal fraction declined, but it remained at a high level in the total protein fraction. This result suggests that the majority of the RcDGAT protein is present in microsomes from 12 to 40 DAP, but after 40 DAP a significant amount of the protein starts to accumulate in fractions other than microsomes. The specific particulate fractions with which RcDGAT may be associated and its biological function are under investigation.

DGAT activity was predominantly a function of the level of RcDGAT protein. Our previous studies showed that there were multiple phosphorylation sites based on functional motifs and critical amino acid residues in the deduced amino acid sequence of RcDGAT (11). It is possible that RcDGAT activity is controlled posttranslationally. It was known that most of the DGAT activity was recovered from the 100,000 × *g* pellet, so this microsomal pellet was chosen for further characterization. DGAT activity was measured in an *in vitro* assay with microsomal membranes isolated from maturing seeds. Enzyme activity was barely detectable before 19 DAP, increased to a significant amount at 26 DAP, reached a maximum at 40 DAP, then quickly decreased until 54 DAP (Fig. 2A). These changes in RcDGAT activity closely correlate with the expression pattern of RcDGAT proteins from microsomes (Figs. 2A and 2B). This result suggests that there is no posttranslational control in RcDGAT activity, which is consistent with the regulation of DGAT1 activity from mouse adipocytes (26). As indicated in Figure 2B, a majority of the RcDGAT proteins are present as partial products (50 kDa) of the entire DGAT protein (60 kDa) in microsomes of develop-

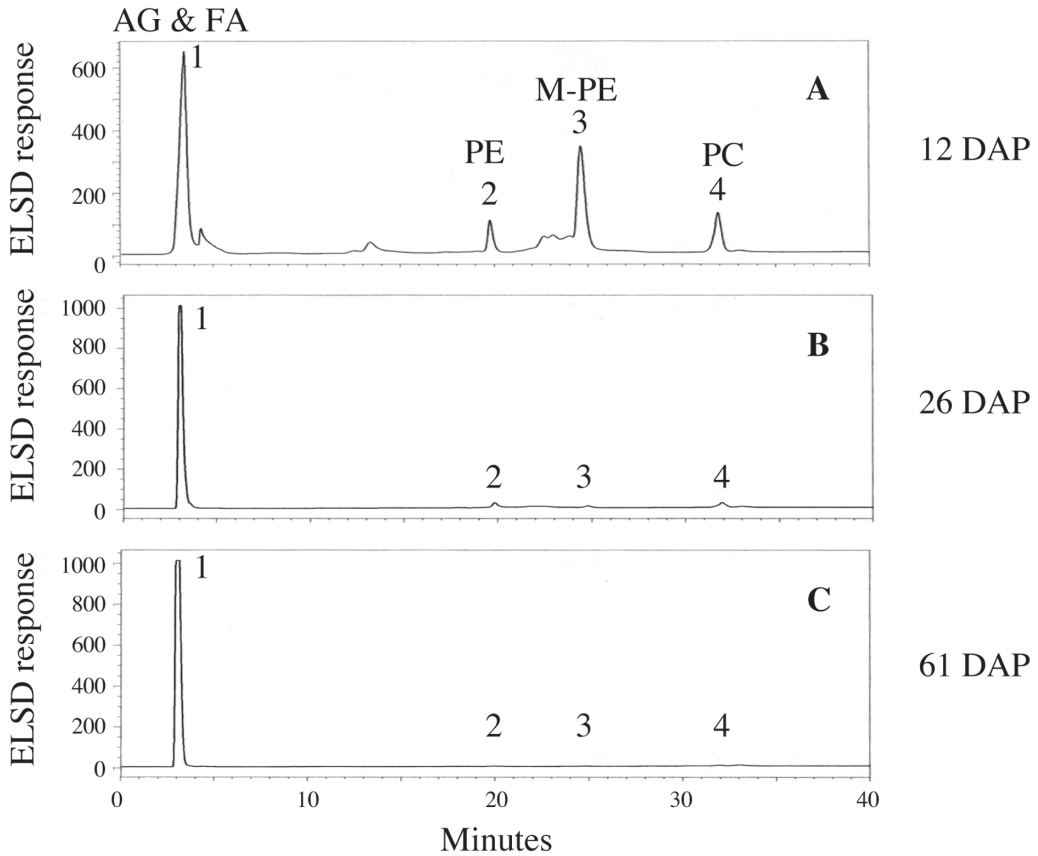

FIG. 4. Separation of lipid classes of total lipid (100 μg) extracted from castor seeds using a silica HPLC and ELSD detection system. (A) Lipid extracted from seeds at 12 DAP; (B) lipid extracted from seeds at 26 DAP; (C) lipid extracted from seeds at 61 DAP. (1) Acylglycerols (AG) and FFA; (2) PE; (3) *N*-methyl-PE (M-PE); (4) phosphatidylcholine (PC). Other peaks are unknown. For other abbreviation see Figure 1.

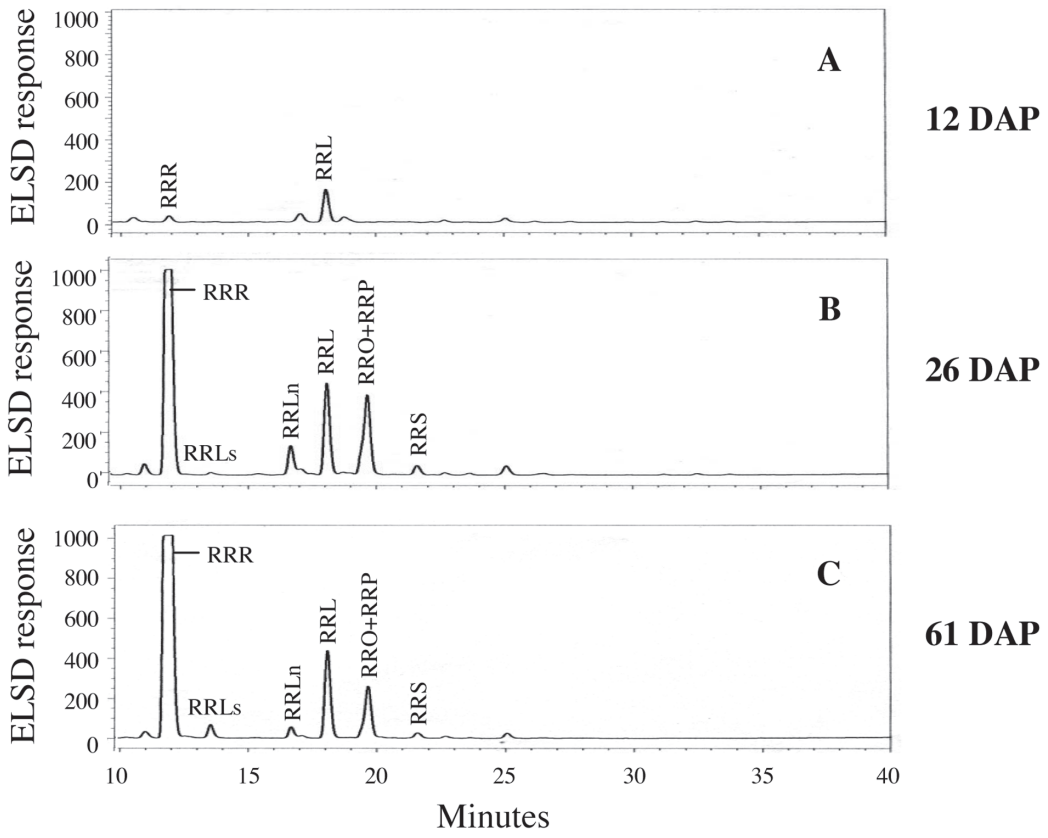

FIG. 5. Separation of molecular species of triacylglycerol (TG) (isolated from 100 μg of lipid) from castor seeds using C18 HPLC and ELSD detection system. (A) TG isolated from seeds at 12 DAP; (B) TG isolated from seeds at 26 DAP; (C) TG isolated from seeds at 61 DAP. For HPLC conditions, see the Experimental Procedures section. R, ricinoleic acid; L, linoleic acid; Ls, lesquerolic acid; Ln, linolenic acid; O, oleic acid; P, palmitic acid; S, stearic acid; for other abbreviation see Figure 1.

ing seeds. The same microsomal preparations were used when measuring the RcDGAT activities, suggesting that the cleavage of the 10-kDa peptide (possibly N-terminal hydrophilic region) from the intact protein does not seem to destroy the enzyme activity. It is possible that cleavage of the 10-kDa peptide represents a form of posttranslational regulation. Recently, DGAT2 was identified from animals, fungi, and plants (10,27). It is unclear whether a DGAT2 is present in castor seeds. We predict that the predominant activity in developing seeds of castor may come from DGAT1, based on the correlation between the total DGAT activity and the RcDGAT protein expression profile. However, we cannot exclude the possibility that DGAT2 is present, because the assay conditions have been optimized for DGAT1.

Developmental changes in oil accumulation of castor seeds. To determine the functionality of the changes in RcDGAT activity during seed development, oil content at each stage was determined. As shown in Figure 3, the onset of oil accumulation was coincident with the appearance of DGAT activity. The maximal rate of lipid accumulation occurred when the DGAT activity reached its peak at 40 DAP. The rate of oil accumulation slowed after 40 DAP, and that was the time at which the activity started to decrease. The tight correlation between DGAT activity and oil deposition also has been reported by other groups (28,29). For many years, acyl-CoA-dependent DGAT was thought to be the only enzyme directly involved in TG biosynthesis. Recently, an acyl-CoA-independent enzyme called phospholipid:diacylglycerol acyltransferase (PDAT) was identified in plants and yeast (30). This enzyme catalyzes the transfer of acyl groups from the *sn*-2 position of the major phospholipids to DG, thus forming TG and lysophospholipids. Based on the correlation of activity with oil accumulation, our results suggest that DGAT using acyl-CoA as a substrate for acyl transfer to DG is the main pathway for synthesis of TG, but we have not eliminated the possibility that PDAT may also display enzymatic activity levels similar to DGAT during seed development of castor.

Developmental changes in lipid classes and TG contents. Castor seed contains high levels of hydroxy FA in its TG. Much of the research concerning unusual plant FA has been focused on the identification of new structures. Little is known about how the synthesis and incorporation of these unusual FA is regulated. Because of the importance of maintaining membrane integrity, accumulation of unusual FA produced by plants is usually restricted to the TG. Plants that produce such

unusual FA must have mechanisms to ensure the removal of the modified FA from the membrane lipids. One of the possibilities is that these seeds may have selective acyltransferases to incorporate unusual FA into the neutral lipid fraction. Before testing this mechanism, we studied the FA distribution and TG accumulation and compared them with the expression profile of RcDGAT protein during seed development. The same amounts of lipids extracted from each stage were separated into lipid classes by silica HPLC as shown in Figure 4. Besides acylglycerols (AG), there were considerable amounts of PC and PE present in seeds at earlier developmental stages (before 19 DAP). At 26 DAP, the relative amounts of PC and PE in seeds dropped to very low levels. After 26 DAP, the FA were almost exclusively in AG. For analyses of TG composition and content during seed development, the AG fractions collected after silica HPLC were separated by C18 HPLC and quantified using ELSD. Figure 5 shows the separation of major molecular species of TG isolated from different developmental stages. The contents of these TG are shown in Table 1. The percentage of ricinoleate- containing TG in oil extracted from seeds at 19 DAP was only about 7, but it rapidly increased to 77 at 26 DAP. The relative amount of triricinolein in oil increased 53 times from 19 DAP to 26 DAP, but it increased only 1.32 times from 26 DAP to 61 DAP. The data shown here suggest that the time between 19 and 26 DAP is a critical stage for castor oil biosynthesis and that the significant switchover in lipid metabolism coincides with the obvious increase of RcDGAT protein and activity in this period.

We have examined the regulation of RcDGAT expression and function during seed development. We demonstrated that RcDGAT expression was regulated at a posttranscriptional level, since accumulation of transcript greatly precedes detectable translation. RcDGAT activity and its effect on the rate of TG accumulation depended primary on the levels of RcDGAT protein. We monitored changes of the relative amounts of different lipid classes in developing seeds of castor. A dramatic increase of TG and decrease of PC and PE were observed in seeds from 19 to 26 DAP. We also found that the major increase of the relative amount of triricinolein in oil happened at this period of time (increase from 1% at 19 DAP to 53% at 26 DAP). These noticeable changes closely correlated with the increase of RcDGAT protein and activity.

ACKNOWLEDGMENTS

This work was supported in part by ARS CRIS Project 5325-21000-006-00D, ARS Research Associate Program, and a cooperative agreement with Dow Chemical Company (Midland, MI).

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[Received September 16, 2004; accepted November 8, 2004]